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Androgens and the androgen receptor (AR) play a critical role in the development and progression of prostate cancers. The majority of prostate cancers initially respond to endocrine treatment (androgen dependent), but eventually become androgen independent that prove fatal. It appears that a functionally active AR may contribute to the progression of androgen-independent prostate cancers. Understanding the signaling pathways that regulate androgen-dependent and -independent activation of AR mediated transcription would provide valuable information for finding an ultimate cure for this disease. We have proposed to identify the signaling components that regulate AR activity using a novel retrovirus-mediated genetic system, and to understand the mechanism of how the identified factors control AR activity. To this end, we have established and optimized our retrovirus-mediated genetic screen approach. The retroviral vectors to be used have been improved which will allow high-efficiency mutagenesis and ease of manipulation and analysis. We have also made significant progress towards optimizing our screening strategies and establishing a suitable cell line for the genetic screen. With these tools in hand, we are in the process of performing the genome wide genetic screen to identify genes that are important for androgen receptor signaling and study how they may contribute to the progression of prostate cancers.

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Introduction

Normal prostate development and the evolution of prostate cancer depend critically on androgens and their receptors (1). Androgens exert their biological function by activating gene transcription through the androgen receptor (AR), a member of the steroid nuclear receptor superfamily (2). For prostate cancer patients, treatments normally begin with endocrine treatment, either by inhibition of AR/androgen binding or deprivation of androgens, which can result in tumor regression. In most prostate cancer patients, however, the tumors eventually become androgen independent (1, 3, 4, 5). An important step towards prostate cancer remedies therefore is the understanding of the signaling pathways that regulate androgen-dependent and -independent activation of AR-mediated transcription.

The overall objective of this proposal is to understand the molecular machinery that regulates AR transcription in normal vs. tumor prostate cells. We propose to utilize a genetic method to screen for genes that regulate AR activity in cultured cells through high-efficiency mutagenesis using Enhanced Retroviral Mutagens (ERM) (6). A genetic screening method will be established to systematically search for genes that modulate AR activity *in vivo* using the PSA (prostate-specific antigen) enhancer and ARE as a reporter. How the identified genes regulate the transcriptional activity and subcellular localization of AR in the context of chromatin will then be investigated.

Body

Task 1. **To isolate genes that are invovled in regulating the activity of androgen receptor**

We proposed to isolate genes that are involved in regulating the activity of androgen receptor. Specifically, clones of cells that can escape androgen-dependent caspase-mediated apoptosis as a result of retrovirus-mediated mutagenesis (through ERM) were to be isolated. Subsequently, the gene loci that have been mutated to confer androgen independence in these clones can be identified.

To accomplish this, we have generated a stable HeLa cell line that expresses the androgen receptor (pCMC-AR). This cell line was then further manipulated to stably express constructs that encoded luciferase, HSV thymidine kinase, and FKBP-caspase driven by ARE. We then tested these stable cells for their abilities to undergo apoptosis, and found that <50% of cells underwent apoptosis in the presence of androgen. Although these cells can be used for the genetic screen, the high level of survival cells in the presence of apoptosis inducing signals will result in high background during the genetic screen. It will significantly increase not only the number of clones that have to be analyzed, but also the chance that authentic mutant clones would be missed.

We therefore went on to construct new vectors to circumvent the high background problem. These new constructs contain 2xGRE upstream of GFP-NTR (Green fluorescent protein-nitroreductase) (7). Cells that successfully take up this new construct would express GFP upon androgen stimulation, while the NTR allows drug selection against GFP-NTR expression. This scenario was tested in both the stable AR-expressing HeLa cells and the LNCaP cells that contain AR. Two separate constructs have been generated for expression in HeLa and LNCaP cells respectively. The expression constructs were first tested in transient transfection assays (Table 1).

Table 1. Percentage of GFP+ cells upon androgen (R1881) treatment in HeLa cells

	-R1881	+R1881
pLCGGN + pCRAR	<1%	~95%
pGGN + pCRAR	<1%	~95%

The above results showed that the constructs were working as intended. We then went on to generate stable HeLa cells expressing both constructs. To ensure that no cells were expressing GFP before R1881 induction, the cells had been analyzed beforehand. After obtaining stable clones (G418 selection), we tested for their ability to undergo apoptosis in the presence of metronidazole (MN) (Table 2). We also confirmed that the majority of the stable cells expressed GFP in response to R1881, similar to the data obtained from the transient transfection experiment (Table 1).

Table 2. Response of HeLa cells stably expressing NTR to metronidazole

MN (mM)	0.1	0.5	1	5	10	15
apoptosis	-	-	-	-	+	+

We then selected two populations of cells that were resistant to MN treatment: GFP positive (GFP+) or GFP negative (GFP-). Both types of cells were used in the ERM-mediated genetic screen. Figure 1 illustrates the selection method.

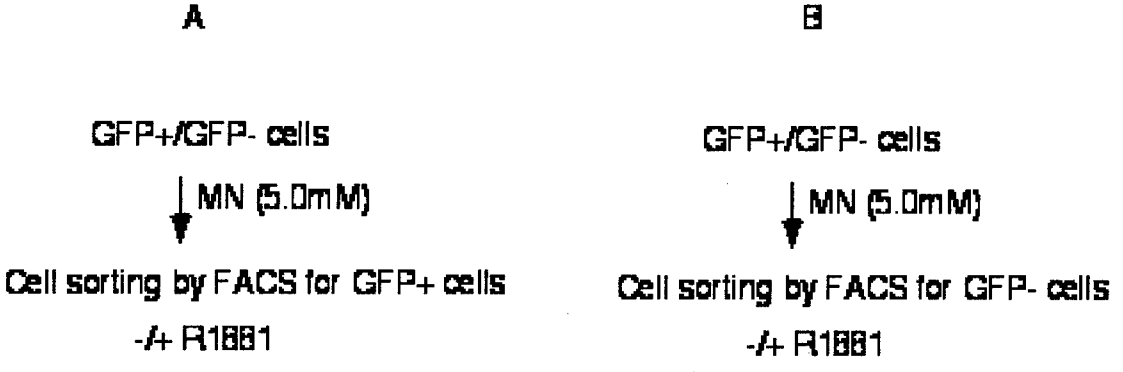

Figure 1. Flowchart for selection of stable cells resistant to MN

In our pilot experiment for the genetic screen, we used the stable HeLa cell line that expresses the androgen receptor (pCMC-AR) as well as GFP-NTR (7). NTR allows drug selection against GFP-NTR expression. In the presence of metronidazole (MN), NTR-expressing cells will die. The rationale was that we would sort GFP^+ and GFP^- cells respectively after MN treatment. However, we found that it was impossible to isolate GFP^- cells after androgen

treatment. Theoretically, androgen stimulated GFP expression should be transient. However, we found that the HeLa-GFP cells remained GFP+ even days after the removal of R1881.

We concluded that cell death could no longer be used as one of the criteria for isolating mutant clones. We therefore modified our screening strategy. As delineated in Figure 2, we took advantage of the tetracyline-responsive promoter already engineered into the ERM vectors (6). This promoter is turned off in the presence of tetracycline (tet-off). Briefly, we sort by FACS GFP^{hi}-AR HeLa cells. These cells are then used for infection with the ERM viruses. Successful ERM integration into the genome may result in lowered GFP expression if the ERM activates a repressor of the AR. We then will sort for GFP^{lo} expressing cells for further expansion. These GFP^{lo} cells will be maintained in the presence of tetracycline, which should turn off the tet-off promoter and lead to decreased levels of the putative repressor. Such a decrease will in turn alleviate the repression of GFP expression and results in an increase in GFP levels. Clones that exhibit such a shift in GFP expression (from GFP^{hi} to GFP^{lo}, and then to GFP^{hi}) will be selected and analyzed to clone the gene targeted by ERM.

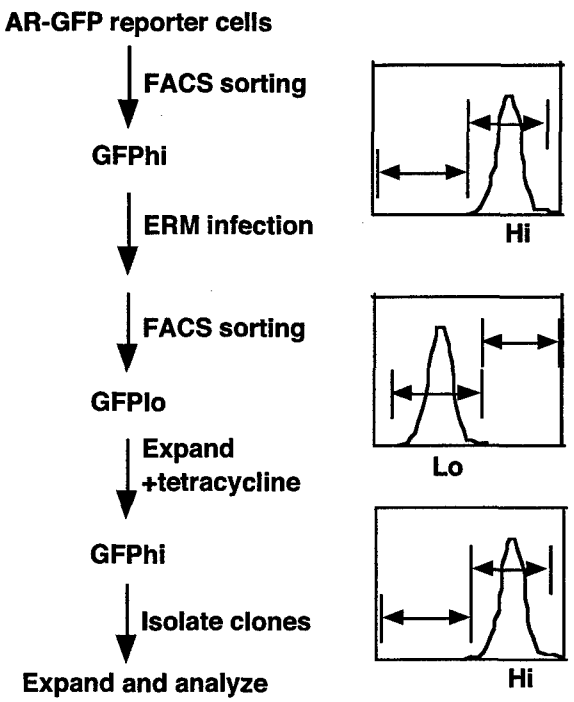

Figure 2. ERM-mediated genetic screening strategy.

We carried out the genetic screen as described, but encountered an unexpected result. After one round of Androgen stimulation, the cells no longer respond to Androgen, indicating that the ARE-driven reporter system is only appropriate for assaying transient activation, but not suitable for genetic screens.

Task 2. **To biochemically characterize the candidate prostate cancer genes identified in Task 1.**

The unexpected technical problems that we encountered in Task1 severely hindered our ability to carry out a large-scale genetic screen as originally planned. As we are still working

through these difficulties, we are unable to identify novel genes that may regulate AR function in human cells.

Key Research Accomplishments

Establishment and improvement of the ERM genetic screen approach
Generation of multiple androgen-responsive reporter cell lines
Improvement of the cells used for the ERM screen
Discovering that ARE-reporter sustains the expression of the reporter genes after first round of Androgen stimulation

Reportable Outcomes

One major outcome is our improvement of the efficacy and efficiency of the genetic screen. We have generated second- and third-generation retrovirus-based genetic screen vectors that should provide high efficiency mutagenesis coupled with makers and epitopes that are easier for analysis and manipulation. Another important outcome is our optimization of the screening strategies to allow for successful genetic screen.

1) Cell lines: Hela-CMV-AR; Hela -CMV-AR/TK; Hela-ARE-GFP-NTR
2) Vectors: pCMV-AR, ARE-luciferase, ARE-TK, ARE-GFP-NTR, ERM vector systems
3) Employment and research opportunities:
   A technician Amin Safari was supported by the grant.

Conclusions

In summary, we have generated multiple stable cell lines that would be useful for further study of AR signaling pathways. And we have further improved our genetic screen approach for high-efficiency mutagenesis. Our progress was severely hampered because of technical difficulties in generating a suitable cell line to use for long-term screens, even after our continuous efforts in modifying our screening strategies. Interestingly, we found that the ARE-GFP cells remained GFP positive after one round of Androgen stimulation. It suggested that the molecular mechanisms to shut down the ARE-gene expression (utilized by endogenous AR responsive genes) may be compromised in this system. This result has pointed a new direction for us to investigate in the future and may prove invaluable in our quest for the cure of prostate cancers.

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